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Improved Photochemotherapy of Malignant Cells With Daunomycin and the KTP Laser

Marcos B. Paiva, MD, PhD,[1]* Romaine E. Saxton, PhD,[2] Ines P. Graeber, MD,[3] Neva Jongewaard, BS,[1] Adrien A. Eshraghi, MD,[4] Michael J. Suh, MS,[5] Woo H. Paek, BA,[1] and Dan J. Castro, MD[1]

[1]Division of Head and Neck Surgery, Department of Surgery, UCLA School of Medicine, Los Angeles, California 90095-1794
[2]Division of Surgical Oncology, Department of Surgery, UCLA School of Medicine, Los Angeles, California 90095-1794
[3]Division of Head & Neck Surgery, Free University of Health and Science, Benjamin Franklin Hospital, D-12200 Berlin, Germany
[4]Department of Head and Neck Surgery, Groupe Hospitalier Pitie-Salpetriere, Paris, France
[5]The Chicago Medical School, Chicago, Illinois 60064

Laser photochemotherapy of malignancies may become an effective palliative treatment for advanced had and neck cancer using light-sensitive, chemotherapeutic drugs activated in tumors via interstitial laser fiberoptics. Previously, it was reported that cultured human P3 squamous cells incubated 2 hours with daunomycin (Dn) exhibited tenfold enhanced cytotoxicity after exposure to argon laser light at 514 nm. This short-term uptake leads to drug localization in cytoplasmic and membrane sites prior to nuclear accumulation and daunomycin topoisomerase inhibition. In the current study phototoxicity of Dn-sensitized human cancer cells was tested using broad-spectrum white light compared to monochromatic green-wavelength light. Drug uptake and laser energy levels were optimized for maximum synergy. To test light-enhanced chemotherapy in vitro, the kinetics of cell uptake and toxicity of daunomycin was measured at 1, 2, and 5 μg/ml in three human tumor cell lines: P3 squamous-cell carcinoma, M26 melanoma, and TE671 fibrosarcoma. After 2 hr Dn uptake, all cell lines were tested for phototherapy response by exposure to 300- to 900-nm visible light from a xenon lamp or monochromatic 532-nm green light from a KTP laser. When the KTP laser output was varied from 0 to 120 Joules in Dn-sensitized tumor cells, a linear phototherapy response was seen with energy as low as 12 J inducing drug phototoxicity. These results provide evidence that daunomycin cytotoxicity is enhanced when exposed to 532-nm laser illumination in the three tumor types tested and confirm that the response is related to both energy level and drug dose. Lasers Surg. Med. 23:33–39, 1998. © 1998 Wiley-Liss, Inc.

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Correspondence to: Marcos Paiva, MD, PhD, 31-24 Rehab Building, 1000 Veteran Ave., UCLA School of Medicine, Los Angeles, CA 90095-1794.

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INTRODUCTION

Laser chemotherapy is an alternative cancer treatment that uses a monochromatic light delivered via external and/or interstitial fiberoptics to enhance the “killing” threshold in tumors containing light- or heat-sensitive conventional chemotherapeutic drugs. Combining intratumor chemotherapy with laser energy delivery via interstitial fiberoptics should be most effective using drugs activated by photothermal energy [1–3]. Adriamycin derivatives are the most common anticancer agents that interact with light to elicit fluorescence, membrane photolabeling, laser activation, and killing of tumor cells [1,4,5]

Imaging-guided minimally invasive surgery is a new concept that uses ultrasound and/or high-speed magnetic resonance imaging to guide various energy sources such as lasers, radio frequency, ultrasonic, or cryotherapy devices for treatment of deep-seated tumors and monitoring of tissue changes during energy depositon [6]. Unlike open surgical procedures in which there is direct visual control of the field, percutaneous techniques require imaging modalities to define the anatomy of adjacent neurovascular structures while providing precise guidance for minimally invasive access to tumors and continuous monitoring of the tissue ablation process. Although interstitial laser therapy (ILT) is less invasive than surgical resection, it appears that ILT as a single treatment modality will fail in those cases in which tumors encroach upon heat-sensitive vital structures [7]. An alternative approach is to deliver laser photothermal emissions to tumors after uptake of light- and heat-sensitive anti-cancer drugs, allowing therapy at reduced thermal energy and elevated intratumor drug levels [8–10].

The objective of the current study was to compare tumoricidal effects of broad-spectrum white light and monochromatic KTP laser emissions at 532 nm in cancer cells sensitized with daunomycin (Dn). Laser photochemotherapy was tested in three cultured human tumor lines including a squamous-cell carcinoma, a melanoma, and fibrosarcoma. Laser energy titration in Dn-sensitized P3 squamous carcinoma cells showed that the cytotoxic response was related to both light fluence and drug dose. The results provide further evidence for the concept of combining light-sensitive drugs with laser photothermal energy as a useful cancer treatment modality.

MATERIALS AND METHODS

In Vitro Culture of Human Cell Lines

The human tumor cell lines UCLA-SO-P3 squamous-cell carcinoma, TE671 fibrosarcoma, and M26 melanoma were cultivated in tissue culture medium (RPMI 1640) supplemented with 10% fetal bovine serum, 10 mmol/liter HEPES buffer, 50 μg/ml Gentamicin, and 50 μg/ml Fungizone (amphotericin B) at 37°C in a humidified NAPCO CO_2 incubator. The cells were plated in 75-cm^2 tissue culture flasks, and complete RPMI 1640 medium was changed at 3-day intervals. Cells were harvested after reaching 80–100% confluency by detachment with 0.25% trypsin, pelleted for 5 minutes at 500*g* in a refrigerated centrifuge, and resuspended in culture media. Viable cells were counted using a microscope by the trypan blue exclusion assay with a calibrated hemocytometer slide.

Daunomycin Uptake

Daunomycin (Sigma, St. Louis) stock solution at 1 mg/ml was dissolved in RPMI 1640 complete medium before use. P3, M26, and TE671 cells (10^6/ml) were incubated in microcentrifuge tubes with 5 μg/ml Dn at 37°C to assess drug uptake. At each time point (30, 60, 90, 120, and 180 minutes) tumor cells were washed in Dulbecco’s phosphate-buffered saline solution (PBS) and then dissociated in 3 ml of dimethyl sulfoxide (DMSO, SIGMA, St. Louis, MO) to release daunomcyin. Drug extracted from the tumor cells was measured by quantitation of Dn fluorescence at the emission maximum of 589 nm using a DMF3000 spectrofluorometer (Fluoromax, SPEX Industries, Inc., Edison, NJ). Dn concentrations from 1 to 100 ng/ml in DMSO were used to construct standard curves of fluorescence intensity measured as CPS. Fluorescence in DMSO extracts from the tumor cells was measured at twofold dilutions to obtain CPS values within this range and Dn uptake levels by the cells calculated from the standard curve.

Daunomycin and Light Treatment of Tumor Cells

Dilutions of Dn at 0, 1, 2, and 5 μg/ml final concentration were added to 10^5 tumor cells in 0.5 ml RPMI media and incubated for 2 hr at 37°C. The P3, TE671, and M26 cell samples were pelleted, washed, and resuspended clear medium (Cellgro, Mediatech, VA) containing 10% fetal calf serum without phenol red and placed on ice. Triplicate samples of all three cell lines in tubes were

kept at 4°C in an ice bath and placed in the dark until exposed to the KTP laser or the xenon lamp as described below.

A KTP (potassium titanylphosphate) crystal laser (Laserscope, San Jose, CA) emitting continuous-wave 532-nm green light at 5 W was coupled to a 0.6-mm fiberoptic cable for light exposure. The fiberoptic tip was positioned 5 cm above the rim of each microcentrifuge tube containing tumor cells and aligned so the laser light cone was slightly larger than the inner diameter of the tube. The tubes were immersed in an ice bath and exposed to the KTP532 laser light for 60 seconds. Actual power output at the fiber tip measured with a Coherent Labmaster E light meter was 4.37 W, and power density was calculated to be 6.83 W/cm^2.

White light exposure was performed with a 20A xenon lamp source (ILC Technology, Sunnyvale, CA) located 12 cm above the microcentrifuge tubes. The cells were exposed to the light for 60 seconds, and measured power density was 6.17 W/cm^2. All treated samples were then transferred as triplicate aliquots of 10^4 cells/0.1 ml into separate wells of Costar 96-well microculture plates containing 0.1 ml of RPMI media and incubated at 37°C for 72 hours. Controls included wells of untreated cell samples and cells exposed to Dn alone in the dark.

Drug and KTP Laser Titration

P3 cells (10^5/0.5 ml in RPMI media) were incubated with Dn (0, 5, and 10 μg/ml) for 2 hr at 37°C, pelleted in a centrifuge, and resuspended in clear CELLGRO culture media without phenol red for KTP laser treatment in 1.5-ml microcentrifuge tubes at room temperature. P3 cells were exposed for 60 seconds to the KTP 532 laser at varied energy levels of 0, 0.05, 0.2, 1, and 2 W (0, 3, 12, 60, and 120 J, respectively). All treated cell samples were transferred to triplicate microculture plate wells containing RPMI media as above and incubated for 72 hr.

Cell Toxicity Measurement by MTT Tetrazolium Assay

After 3 days incubation, MTT tetrazolium bromide (Sigma, St. Louis, MO) at 0.5 mg/ml was added to all wells for 4 hr prior to aspiration. The MTT assay measures mitochondrial dehydrogenase activity and is currently among the most reliable methods to assess cytotoxicity after chemotherapy or phototherapy of cultured human tumor cells [11]. After MTT incubation, cells were resuspended in 0.2 ml DMSO to solubilize the blue formazon precipitate and chromophore quantitated at 550 nm with an enzyme-linked immunosorbent assay (ELISA) reader (Molecular Devices, Corp.). All experiments were repeated three times before average values were calculated with standard deviation represented as error bars using EXCEL spreadsheet for both data entry and analysis.

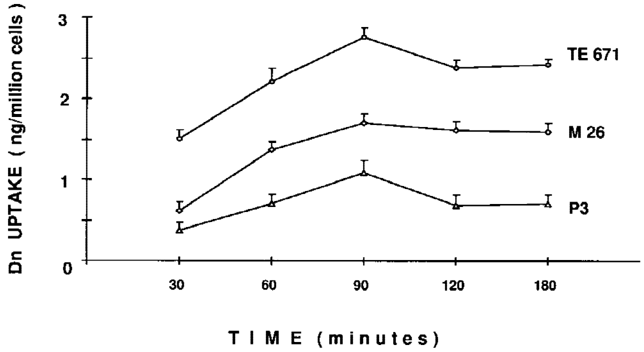

Fig. 1. Daunomycin uptake by P3, M26, and TE671 cells at 5 μg/ml of drug for increasing times (30, 60, 90, 120, or 180 min) measured by fluorescence. Error bars represent standard deviation of average values.

RESULTS

Daunomycin Uptake by Tumor Cell Lines

Drug uptake by the three human tumor cell lines—P3, M26, and TE671—was compared by quantitative fluorescence assays after cell incubation with 10 μg/ml Dn for different time points (30, 60, 90, 120, and 180 min). Fibrosarcoma TE671 cells exhibited threefold higher drug incorporation after 90 min than P3 squamous carcinoma cells as shown in Figure 1. Daunomycin uptake increased progressively with time in all cell lines during the first 90 min of incubation followed by a decline, indicating rapid saturation or tumor cell cytotoxicity leading to an apparent drop in drug incorporation. This rapid uptake of Dn indicates that drug levels were sufficient for both chemotherapy and photosensitization. Increased drug uptake by TE671 compared to P3 suggested that these sarcoma cells might be more sensitive to both phototherapy and Dn cytotoxicity.

Photoactivation of Dn-Sensitized Tumor Cells With 532-nm vs. White Light

The P3 squamous carcinoma cells were found to be more resistant than the M26 or TE671

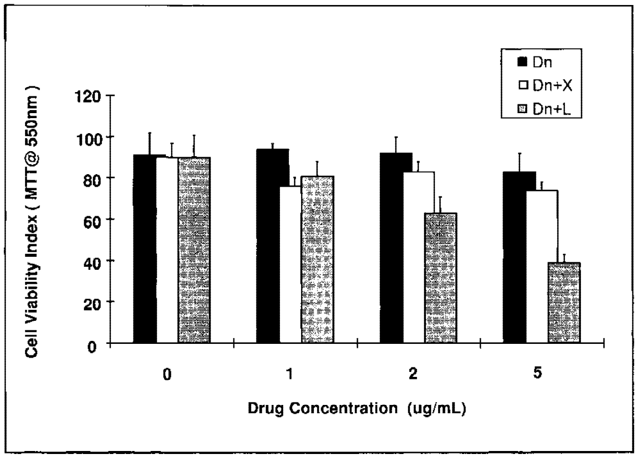

Fig. 2. Viability of P3 squamous-cell carcinoma cells measured by MTT assays after Dn and light exposure at various drug levels.

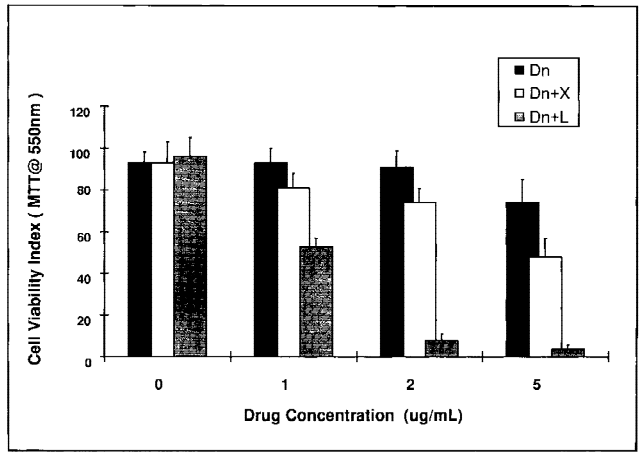

Fig. 3. Viability of M26 human melanoma cells measured by MTT assays after DN and light exposure at increasing drug levels.

cell lines at all Dn concentrations tested after drug alone or Dn phototherapy (Fig. 2). Significant tumoricidal enhancement by the KTP laser light was seen at higher Dn concentrations (2 and 5 μg/ml) with maximum laser light effects observed at 5 μg/ml of Dn, resulting in over 50% increased toxicity compared to drug alone. The KTP laser increased phototoxicity significantly, but the xenon lamp enhancement was marginal as shown in Figure 2.

The most significant daunomycin photoactivation response was observed in the M26 melanoma cells at Dn concentrations of 2 and 5 μg/ml with ten- and 20-fold elevated cell killing, respectively, after exposure to the KTP laser when compared to Dn alone in the absence of light, as shown in Figure 3. This light enhancement effect of Dn also was detected after white light exposure of M26 cells, but clearly the KTP laser induced a more robust phototherapy response (Fig. 3). TE671 fibrosarcomas cells at Dn concentrations of 2 and 5 μg/ml also exhibited elevated phototoxicity with the KTP laser light, increasing cytotoxicity two- and threefold higher than broad-spectrum white light as shown in Figure 4. These photoactivation experiments provide in vitro evidence of improved chemotherapy responses to daunomycin in all three human tumor cell lines tested with consistently higher Dn cytotoxicity seen after cell exposure to the 532-nm laser emissions than to broad-spectrum white light.

Dose Response of Laser and Drug Toxicity

In order to define minimum drug and laser energy levels for combined therapy, P3 squamous carcinoma cells were incubated with Dn at 0, 5, or 10 μg/mL and illuminated with the KTP laser at increasing energy (0, 3, 12, 60, and 120 J). As shown in Figure 5, the results of this experiment revealed that only 12 J of 532-nm light was needed to amplify drug toxicity. A linear cytotoxic response to increased laser energy was seen for P3 cells after incubation with 5 μg/ml Dn. Tumoricidal effects were approximately two-, three-, and eightfold amplified by 532-nm light at 12, 60, and 120 J, respectively, compared to drug in the absence of light. However, P3 cells incubated in the dark with 10 μg/ml of Dn exhibited such high cytotoxicity that an increased response after light exposure was not detected (Fig. 5). The data indicates that 532-nm visible light enhances Dn che-

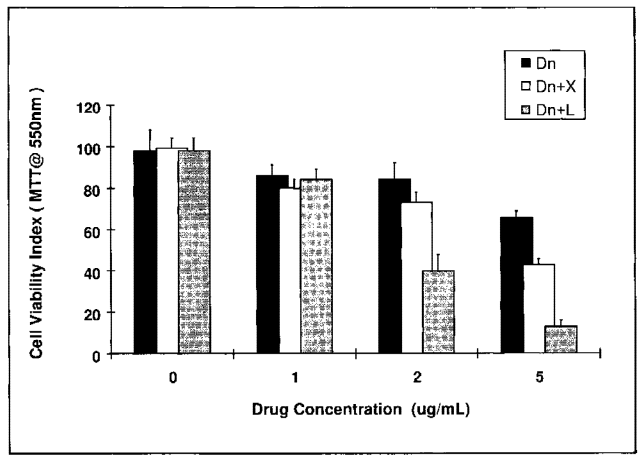

Fig. 4. Viability of TE 671 fibrosarcoma cells measured by MTT assays after Dn and light exposure at increasing drug levels.

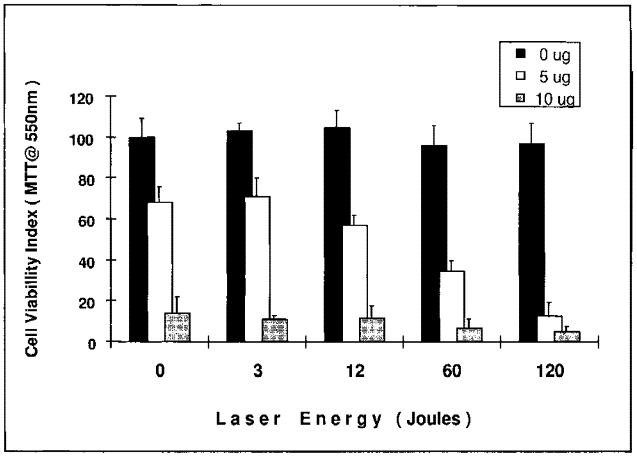

Fig. 5. Viability of P3 carcinoma cells measured by MTT assays after Dn uptake and KTP laser exposure at various drug and energy levels.

motherapy of human tumor cell lines with cytotoxicity dependent on both energy levels and drug dose.

DISCUSSION

Daunomycin is a fluorescent glycosidic antibiotic of the anthracycline group that acts as a DNA topoisomerase II inhibitor with clinical usefulness in the therapy of several types of human cancer [12,13]. Daunomycin initially localizes in the cell cytoplasm and binds to tumor cell membranes at significantly greater levels than Adriamycin, which accumulates in the nucleus [14,15]. The initial distribution of daunomycin in the cytoplasm is followed by slow nuclear localization as observed in studies with normal and tumor cells [14,15]. When exposed to light, tumor cell lipid peroxidation and membrane photolabeling occur immediately after daunomycin uptake [5]. Spin-label experiments also have demonstrated that slow light activation of Dn induces chemical bonding to many different cell membrane proteins [5]. Absorption of photons by aromatic organic compounds increases kinetic energy levels and may excite orbital electrons, leading to chemical reactions as well as inducing light emission as fluorescence, or may transfer energy directly to oxygen molecules, forming singlet oxygen and/or free radical species [16].

Andreoni et al. first reported laser-amplified anthracycline toxicity in vitro in normal rodent cells [16]. Saxton et al. [17] found that carcinoma cells incubated with daunomycin can be killed by nonthermal 514.5-nm argon laser illumination at tenfold reduced drug levels compared to drug alone in the dark. Daunomycin toxicity also was shown to be increased by argon laser illumination of Dn in rat thyroid carcinoma cells with evidence for photoactivation more likely via a type I mechanism involving free radicals rather than type II singlet oxygen production [18].

The current studies revealed that Dn uptake was rapid for all three human tumor cell lines tested, and drug saturation or cytotoxicity was seen by 90 min in each case. Because Dn binds to cell membrane sites, it is an ideal anticancer drug for light-activated lipid peroxidation after rapid uptake when combined with green visible light sources [14–18]. The improved response seen for photosensitization of these cells using the KTP 532 laser compared to broad-spectrum white light is most likely a result of the characteristic blue-green light absorption maxima of daunomycin. Monochromatic 532-nm emissions are close to the Dn molecular excitation peak and provide a more efficient photon flux to pump drug photoactivation. For example, the human carcinoma and fibrosarcoma cells incubated with Dn at 5 μg/ml before exposure to the KTP laser exhibited two- to fivefold elevated cytotoxicity compared to chemotherapy in the absence of light. Although the sarcoma cells had greater Dn uptake, the melanoma cells had more significant KTP laser enhancement of drug toxicity with a robust 20-fold amplified cytotoxicity seen at 5 μg/ml Dn. KTP laser energy titration experiments even with the less drug-sensitive squamous carcinoma cells revealed photoactivation at an energy level as low as 12 J after uptake.

The current studies corroborate and extend previous in vitro experiments that demonstrate that anthracyclines have dual properties as photodynamic agents and as cytostatic anticancer drugs [1,8,16–19]. For example, a recent in vitro study with Adriamycin using explanted human squamous-cell carcinoma showed that exposure to the argon laser at 514 nm led to over 50% increased cytotoxicity at drug levels equal to or above 5 μg/ml compared to chemotherapy alone in the dark [19]. Although the current study provides clear in vitro evidence of daunomycin photoenhancement as an anticancer drug, the demonstration of laser chemotherapy in a tissue culture setting differs in many respects from that expected in vivo with human tumors. Tissue culture experiments are particularly useful in preclinical testing to establish optimal laser fluence and drug dose [20]. However, the KTP laser en-

ergy and Dn drug level also need to be optimized using in vivo model tumors before clinical evaluation in patients.

KTP532 laser output is useful for interstitial applications because it can be delivered through submillimeter optical fibers and can be delivered via a micromanipulator attached to an operating microscope or with various hand-held delivery probes. KTP laser light also has been used surgically after coupling to a bare fiber in both the contact mode and noncontact mode with reported clinical effectiveness in controlling bleeding disorders as well as tongue and soft palate cancer resections in addition to laryngeal cancer treatment [21,22]. KTP laser fiberoptics recently were tested for interstitial treatment of transplanted human squamous-cell tumors after injection of the anthrapyrazole CI-941 [9,10]. This agent was selected as a more photosensitive and less toxic anthracycline-related drug than daunomycin for initial in vivo studies of human squamous-cell carcinoma transplants in nude mice [23,24]. The results showed a significantly improved cure rate of 72% after drug and laser therapy compared to 46% for laser treatment alone [10]. Delayed tumor regrowth and prolonged mean survival was seen after laser chemotherapy compared to either KTP laser alone or CI-941 injection alone. In a related study, Peavy et al. [25] have reported successful treatment of a canine fibrosarcoma by combining Adriamycin with local CO_2 laser application. Previously tumor recurrence had been seen in this animal following either treatment modality alone.

Several groups have shown that anthracycline-related agents and other FDA-approved anticancer drugs including cisplatin can be activated in tumors by light or heat to increase the cytotoxic response of these clinically useful chemotherapy drugs [2,3,25–27]. An advantage of anthracyclines for laser chemotherapy is their dual role as antitumor drugs and as photothermal sensitizers. To improve chemotherapy responses, several approaches can be explored. First daunomycin and other drugs may be used systemically at maximum tolerated dose followed by local tumor site photoillumination via interstitial fiberoptics at low energy to improve outcome and to reduce surgical morbidity. Second, improved therapy response to anticancer agents injected directly into tumors may be possible after laser photothermal activation resulting in reduced local and systemic drug toxicity. This low energy laser chemotherapy approach appears to be more effective for tumor palliation and reduces thermal damage to local normal tissues compared to current photoablative laser therapy.

CONCLUSIONS

Daunomycin uptake by cultured human tumor cells induced direct drug cytotoxicity and amplified tumor killing after photoactivation of the cells with visible green light at 532 nm. Strongly enhanced tumoricidal effects were observed after combined drug and KTP532 laser exposure of the three types of human cancer cells compared to slightly increased cytotoxicity with broad-spectrum white light. Reducing the laser light output resulted in a linear decrease in response, but Dn phototherapy was detectable at laser energy levels as low as 12 J output even in the least sensitive cell line. It remains unclear whether laser chemotherapy results from rapid daunomycin incorporation by cell membranes leading to lipid peroxidation or represents sequential drug and laser tumor targeting via other mechanisms. If daunomycin is confirmed to be effective in vivo by further studies of laser chemotherapy in tumor transplant models and does not induce adjacent normal tissue toxicity, it may provide surgeons with a minimally invasive new treatment for management of advanced cancer of the head and neck.

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